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14. ABSTRACT

The Prolactin (PRL)-Jak2-Stat5 pathway has been described as a key regulator in the normal growth, development, and differentiation of human breast epithelia. Recent evidence from our lab and others has suggested that active Stat5 is a positive predictive marker of prognosis in breast cancer patients and the loss of active Stat5 correlates with a more aggressive disease state. Further, in vitro expression of Stat5 increased differentiation characteristics of human breast cancer cell lines and was able to inhibit invasive characteristics in human breast cancer cell lines. The specific aims of this proposal were designed to further investigate the role of Stat5 in breast cancer progression and metastasis. We hypothesize that active Stat5a suppresses invasion and metastasis of human breast cancer by promoting upregulation of differentiation markers, increasing homotypic adhesion, and inhibiting growth. We aim to test this hypothesis in human breast cancer cell lines both in vitro and in vivo using a novel constitutively active Stat5a construct. We have constructed constitutively active Stat5 mutants and generated adenoviral, lentiviral, and tetracycline-inducible expression systems. We have also generated stable cell lines expressing these constructs. Stable expression of Stat5 in mesenchymal MDA-MB-231 breast cancer cells does not yield consistent results with those seen in transient expression systems, and it is likely that we are not using an appropriate cell line to study Stat5 signaling. We are in the process of optimizing cell lines and assays to measure Stat5 differentiation in a more relevant setting where Stat5 intracellular regulators are more likely to be present and functioning. In addition, we have determined that Stat5a and Stat5b correlate with survival in human clinical breast cancer specimens and pY-Stat5 expression correlates with tumor differentiation. We will extend these studies to determining the contribution of Stat5a and/or Stat5b to differentiation phenotype in vitro.

Table of Contents

Page

Introduction

The Prolactin (PRL)-Jak2-Stat5 pathway has been described as a key regulator in the normal growth, development, and differentiation of human breast epithelia. Recent evidence from our lab and others has suggested that active Stat5 is a positive predictive marker of prognosis in breast cancer patients and the loss of active Stat5 correlates with a more aggressive disease state[1, 2]. Further, *in vitro* expression of Stat5 increased differentiation characteristics of human breast cancer cell lines and was able to inhibit invasive characteristics in human breast cancer cell lines[3, 4]. The specific aims of this proposal were designed to further investigate the role of Stat5 in breast cancer progression and metastasis. We hypothesize that active Stat5a suppresses invasion and metastasis of human breast cancer by promoting upregulation of differentiation markers, increasing homotypic adhesion, and inhibiting growth. We will test this hypothesis in human breast cancer cell lines both *in vitro* and *in vivo* using a novel constitutively active Stat5a construct. In addition, we aim to determine if Stat5a and/or Stat5b contribute to the differentiation phenotype *in vitro* and by correlating Stat5 expression and differentiation in human clinical breast cancer specimens.

Body

To facilitate investigation of the aims proposed, novel constitutively-active Stat5a mutants (ca-Stat5a) were designed to enhance the effects of Stat5a activation, particularly in breast cancer cell lines lacking appreciable PRL-Stat5 activation and signaling. Stat5a-S710F was previously described to be constitutively active in hematopoietic cell lines[5]. We constructed adenovirus and tetracycline-inducible wildtype and S710F Stat5a (Figure 1a and 1b). Although we observed moderate constitutive activity of Stat5a-S710F in the absence of prolactin in human breast cancer cell lines (data not shown), we attempted to identify a construct with a higher level of constitutive activity. Serine 725 and serine 779 have been previously reported by our lab to be inhibitory Stat5a serine phosphorylation sites and mutation of both Ser725 and Ser779 to alanine, resulted in a Stat5a construct with increased sensitivity to prolactin[6]. In

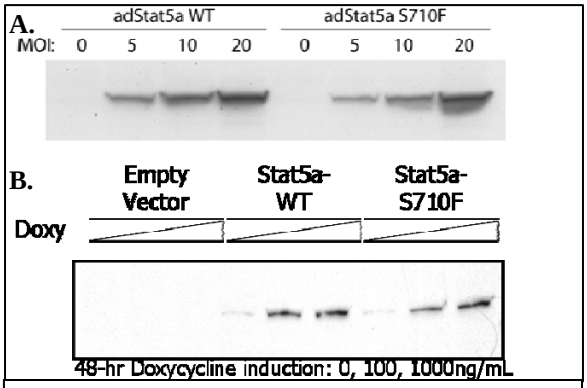


Figure 1. Stat5 expression systems. **A.** Increasing MOI of adenovirus expressing wildtype or ca-Stat5a in T47D. **B.** Tetracycline-inducible wildtype and ca-Stat5 expression in T47D.

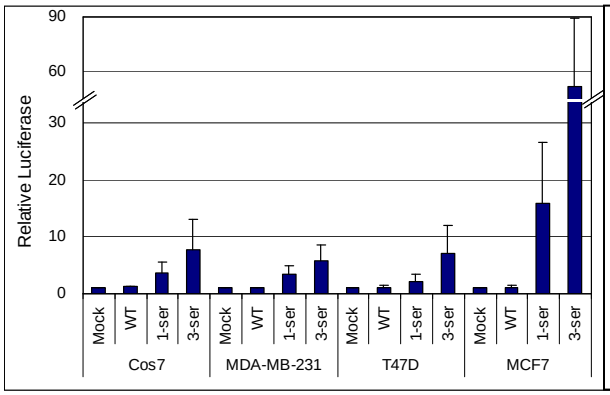


Figure 2. Stat5a-3ser has increased PRL-independent transcriptional activity. Four cell lines were transfected with Stat5a-WT, -1ser or -3ser, hPRLR, and a Stat5a-repsonsive reporter construct. Cells were serum-starved in the absence of PRL. Luciferase expression was measured and normalized to mock cells.

transient expression systems, we identified that combination of S710F with S725A and S779A (Stat5a-3ser) resulted in a construct with at least two times more constitutive activity than the S710F mutation alone (Figure 2) and was hyper-phosphorylated in response to prolactin compared to Stat5a-WT or Stat5a-S710F (data not shown). Stat5a-3ser is also constitutively phosphorylated in the absence of PRL-stimulation (Figure 3) and is more sensitive to low doses of PRL than Stat5a-WT (data not shown).

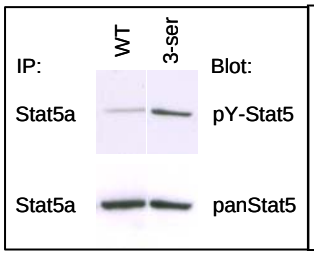


Figure 3. Stat5a-3ser is constitutively phosphorylated in the absence of PRL. MDA-MB-231 cells were transfected with Stat5a-WT or 3ser and serum-starved in the absence of PRL.

In order to study the invasion-suppressive role of Stat5, we proposed to use invasive MDA-MB-231 cells. We stably expressed Stat5a-WT or Stat5a-3ser in MDA-MB-231 breast cancer cells using a lentiviral system (Figure 4). However, initial studies *in vitro* studies have revealed that stable expression of Stat5 in MDA-MB-231 does not respond consistent with responses seen in transient expression. We have noticed a suppression of basal ca-Stat5 activity except when the PRL receptor is co-expressed. We also observed that inducible Stat5 was absent in wildtype Stat5 expressing cells, even in the presence of the PRL receptor (Figure 5). MDA-MB-231 are believed to arise from a mesenchymal cell type, whereas Stat5 is predominately active in luminal epithelial cells. We hypothesize that MDA-MB-231 may not have the intracellular machinery necessary for adequate and appropriate PRL-Stat5 signaling. We are currently investigating additional cell lines that may have more appropriate responses to PRL while still allowing us to study anti-invasive properties.

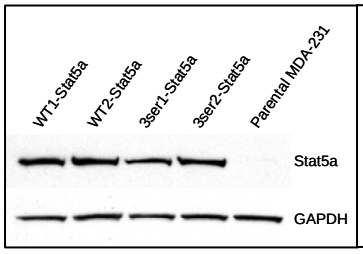


Figure 4. Stable Stat5a expression in MDA-MB-231. MDA-MB-231 were infected with lentivirus expressing indicated Stat5a construct.

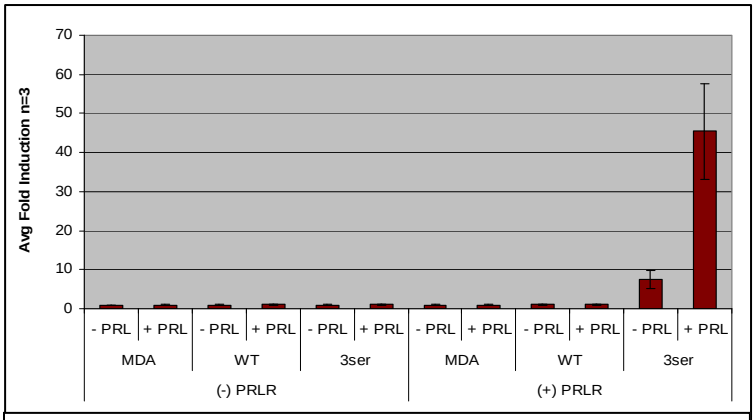
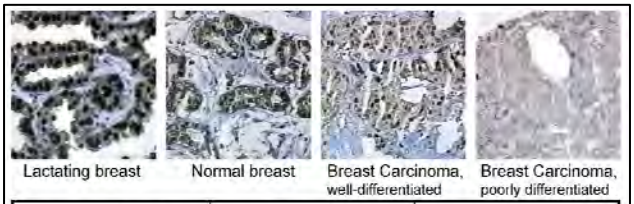


Figure 5. MDA-MB-231 stably expressing wildtype and ca-Stat5 do not respond in transcriptional assays as expected based on transient *in vitro* studies. MDA-MB-231 stable cell lines were co-infected with control adenovirus or PRLR adenovirus and a Stat5-responsive luciferase reporter. Cell lysates were measure for luciferase expression and normalized to controls.

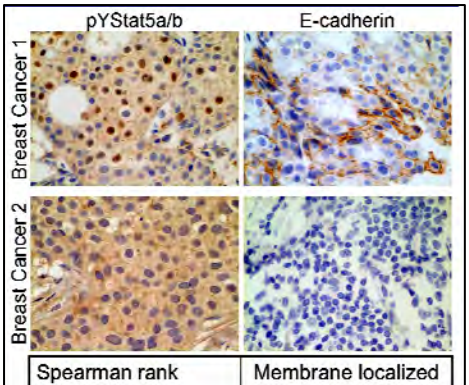
To further understand the role of Stat5 in breast cancer differentiation and suppression of invasion, we aimed to evaluate the clinical relevance of Stat5 expression in human breast cancer. We further analyzed the clinical breast cancer specimens from our previously published survival study[1] and found a positive correlation between nuc-pYStat5 and tumor differentiation and an inverse correlation between nuc-pYStat5 and mitotic rate

(Figure 6). Based on *in vitro* data, we hypothesized that one mechanism for Stat5-induced differentiation may be by maintaining E-cadherin at the cellular membrane. Thus, we performed a retrospective follow-up analysis on 400 node-negative breast cancer specimens with corresponding outcome data provided by the National Cancer Institute's Cooperative Breast Cancer Tissue Resource (CBCTR). We found a positive correlation between nuc-pYStat5 and membrane localized E-cadherin (Figure 7).



Spearman rank correlation	Tumor differentiation	Mitosis rate
Stat5 pTyr *rho*	+0.358	-0.380
Sig. (P)	<0.001	0.003
N	477	380

Figure 6. Nuclear localized, tyrosine phosphorylated Stat5 correlates with differentiation of human breast cancer. A breast cancer tissue array of 500 breast cancer specimens was analyzed for pYStat5 by immunohistochemistry. Nuc-pYStat5 levels were correlated with tumor grade and differentiation as well as mitotic rate.



Spearman rank correlation	Membrane localized E-cadherin
Nuclear pY-Stat5 *rho*	+0.14
Sig. (P)	0.008
N	363

Figure 7. Membrane localized E-cadherin positively correlates with nuclear localized, tyrosine phosphorylated Stat5. Whole tissue sections from 363 breast carcinomas were analyzed by immunohistochemistry for E-cadherin and pY-Stat5.

A limitation of nuc-pYStat5 IHC studies is that the phosphotyrosine-Stat5 antibody does not discriminate between pStat5a and pStat5b. Therefore, we analyzed total levels of nuclear Stat5a and Stat5b and performed survival analysis on the 400 CBCTR samples. We found that both nuclear Stat5a and nuclear Stat5b correlated with favorable breast cancer prognosis (Figure 8). Finally, these samples further validated previous results indicating that nuc-pYStat5 is a significant favorable prognostic marker[1]. Based on our clinical data, we suggest that nuc-pYStat5 is a predictive marker of breast cancer survival and warrants further investigation into the mechanisms of the loss of nuc-pYStat5 and consequential loss of cellular differentiation that may promote invasion and metastasis.

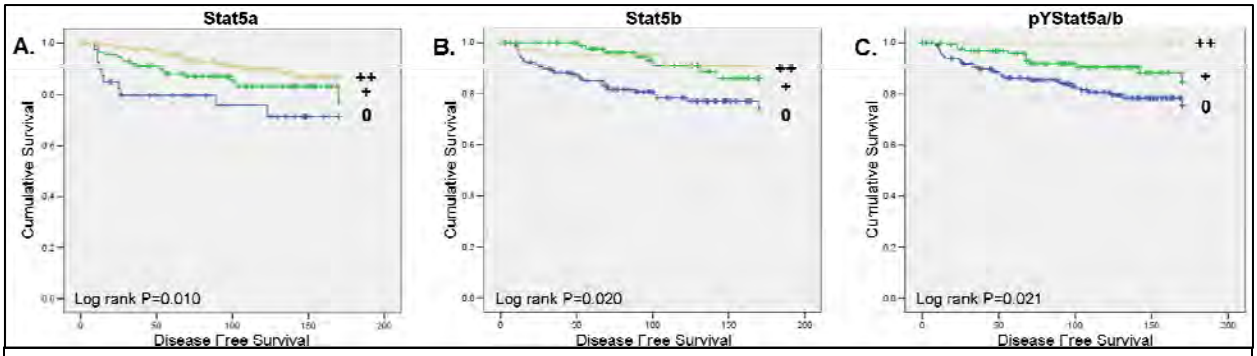


Figure 8. Nuclear Stat5 predicts favorable prognosis in patients with node-negative breast cancer. Formalin fixed, paraffin-embedded sections from 400 node-negative breast cancers with outcome data were analyzed by for nuclear Stat5a, Stat5b and pYStat5a/b by immunohistochemistry and correlated with patient survival. **A.** Stat5a **B.** Stat5b **C.** pYStat5a/b. Yellow: high expression; Green: medium expression; Blue: no expression.

Key Research Accomplishments

- Generated Stat5a and ca-Stat5a MDA-MB-231 stable cell lines.
- Characterized MDA-MB-231 stable cell lines.
- Observed a positive correlation between active nuclear Stat5 and tumor differentiation.
- Identified a positive correlation between active nuclear Stat5 expression and E-cadherin.
- Established that nuclear Stat5a and Stat5b positively correlate with breast cancer survival.

Reportable Outcomes

Abstracts:

Ryder A, Witkiewicz A, Tran T, Rui H. (2008) Nuclear-localized, tyrosine-phosphorylated Stat5 correlates with differentiation in human breast cancer. Gordon Research Conference on Prolactin and Growth Hormone Family.

Conclusion

In summary, we have identified, constructed and characterized constitutively active Stat5a mutants that are tyrosine phosphorylated and transcriptionally active in the absence of prolactin stimulation. Work in progress is focused on optimizing cell systems to determine an appropriate cell line and measurable outcomes to test our hypothesis that Stat5 is able to suppress invasion by maintaining differentiation. We have also indentified relevant clinical correlations with Stat5, differentiation and survival in human breast cancer specimens. The outcomes of these experiments will greatly improve the knowledge base concerning the role of Stat5 in breast cancer and will also provide a foundation for research focused on developing differentiation therapies to treat breast cancer.

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Appendices

None.